UNIVERSITI PUTRA MALAYSIA

MOLECULAR STUDIES OF A HIGHLY VIRULENT STRAIN OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) AND PRODUCTION OF VP2 RECOMBINANT PROTEIN

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By

CHONG LEE KIM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, In Fulfilment of the Requirement for the Degree of Doctor of Philosophy

April 2003

Dedicated with love and gratitude
to:

My parents (Mr CHONG HOW KIM and the Late Mrs. TOO KUEN YIN),
husband (Dr. ALAN ONG HAN KIAT),
son (ZACHARY ONG ZHAN XIANG),
and,
brothers and sisters.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Faculty: Veterinary Medicine

The UPM97/61 isolate originated from the field IBD outbreak was identified as a highly virulent IBDV strain based on the sequence and phylogenetic analysis. The VP2 sequence contains amino acid substitutions at positions 222(A), 256(I) and 294(I) which are genetic markers for highly virulent strains. In addition, the serine rich heptapeptide region, S-W-S-A-S-G-S present in all highly virulent strains are also well conserved. Based on the restriction enzyme analysis, UPM97/61 has one *Ssp*I, *Taq*I and *Sty*I restriction sites at nucleotide positions 1011, 833, and 888 which correspond to amino acid residues 294, 235 and 254, respectively, but absent for *Sac*I site which is also one of the characteristic of the highly virulent strain. The sequences alignment of VP3, VP4 and VP5 with other strains of IBDV had showed that the amino acid substitutions at VP4 (685 Asn), VP3 (715 Ser, 761 Asp, 990 Val and

1005 Ala) and VP5 (49 Arg and 78 Ile) could also be used to differentiate the highly virulent phenotype from the less virulent phenotype of IBDV. Phylogenetic analysis of the individual VP2, VP3, VP4 and VP5 protein as well as the whole segment A of IBDV has cluster the UPM97/61 together with the highly virulent strains from Japan (OKYM), UK (UK661), HK46 (China) and ks (Isreal). It was speculated that the Malaysian highly virulent strain might have the same origin as the highly virulent strains isolated in Europe, Japan, China and Isreal. VP2 protein was classed under alpha-beta mixed family. The β-sheet structure formed the main structure of the hypervariable region where the neutralizing epitopes clustered. It was predicted that, this structure allowed intermolecular interaction as well as intra molecular interaction between the viral protein and the antibody via hydrogen bonding. A single-tube RT-PCR method that is time saving, less laborious and does not require much samples was developed and it has the potential to be used as a diagnostic tool. VP2 protein was successfully expressed in a prokaryotic system. The *E. coli* expressed-VP2 protein was able to induced a significant level of ELISA antibody titer that provided 57% protection to the chicken challenge with highly virulent strain against mortality but not against bursal atrophy. The protection could be improved by a careful evaluation of the vaccine dosage, virus dosage, vaccination timing and route of inoculation in the future studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Doktor Falsafah

KAJIAN MOLEKUL VIRUS PENYAKIT BURSA BERJANGKIT (IBDV) YANG SANGAT VIRULENT DAN PEMBANGUNAN PROTEIN VP2 REKOMBINAN

Oleh

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UPM97/61 isolat telah dikenalpasti sebagai virus penyakit bursa berjangkit yang sangat virulen (vvIBDV) berdasarkan analisis jujukan genetik dan filogenetik. Jujukan pada bahagian VP2 mempunyai penggantian asid amino di kedudukan 222(A), 256(I) and 294(I) yang merupakan petanda genetik untuk vvIBDV. Tambahan pula, heptapeptid yang kaya dengan residu serine; (S-W-S-A-S-G-S), yang ada pada semua vvIBDV juga diperlihara pada UPM97/61 isolat. Berdasarkan analisis pencernaan enzim pembatas, UPM97/61 terdapat satu tapak *Ssp*I, *Taq*I dan *Sty*I pada kedudukan nukleotid 1011, 833, dan 888 yang serupa dengan kedudukan asid amino 294, 235 dan 254 masing-masing, tetapi tidak mempunyai tapak *Sac*I. Ini jugalah satu daripada ciri-ciri vvIBDV. Susunan jujukan asid amino untuk protein VP3, VP4 dan VP5 dengan isolat lain menunjukkan bahawa penggantian asid amino pada kedudukan VP4 (685 Asn), VP3 (715 Ser, 761 Asp, 990 Val, 1005 Ala)

dan VP5 (49 Arg, 78 Ile) juga boleh digunakan untuk membezakan vvIBDV daripada virus bursa penyakit yang kirang virulen. Analisis filogenetik untuk setiap protein VP2, VP3, VP4, VP5 dan juga keseluruhan segment A telah meletakkan UPM97/61 pada kumpulan vvIBDV bersama dengan isolate dari Japun (OKYM), UK (UK661), China (HK46) and Israel (KS). Oleh itu, adalah diagakkan bahawa Malaysia vvIBDV berkemungkinan berasal dari tempat yang sama dengan isolate daripada Europe, Japan, China and Israel. Protein VP2 dikelaskan pada famili alpa-beta bercampur. Struktur lampiran-β adalah struktur utama pada bahagian hipervariasi di mana kawasan epitop peneutralan berkumpul. Adalah diramalkan bahawa struktur ini membolehkan penarikan inter-molekul dan juga intra-molekul antara protein virus dengan antibodi melalui ikatan hidrogen. Cara satu-tiub ataupun satu-langkah RT-PCR yang mejimatkan masa and tidak memerlukan banyak tenaga buruh dan sampel telah dicipta. Sistem ini mempunyai potensi untuk dijadikan alat diagnosa. Protein VP2 juga berjaya diekspres pada system prokaryotik. Protein *E. coli* ekspres-VP2 mempunyai keupayaan untuk menggalak pengeluaran antibodi ELISA pada paras yang ketara dan dapat melindungi 57% daripada kematian ayam yang jangkiti oleh vvIBDV tetapi tidak dapat melindungi ayam daripada kerosakan bursa. Perlindungan boleh diperkuatkan lagi dengan megadakan penilaian teliti pada dos vaksin, dos virus, masa vaksinasi dan juga saluran inokulasi untuk kajian pada masa akan datang.

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CHAPTER I

INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious viral disease of chickens caused by IBD virus. This virus destroys the immature B lymphocytes of the bursa of Fabricius, leading to prolonged immunosuppression and increased susceptibility to other diseases (Lasher and Shane, 1994). IBDV can be divided into two serotypes, serotype 1 is pathogenic to chickens whereas serotype 2, isolated from turkeys, is non-pathogenic to chickens (Becht *et al.*, 1988). IBDV of serotypes 1 includes several strains based on their virulence and antigenic variation: classical strains, attenuated strains, antigenic variant strains and highly virulent (hv) strains. Lasher and Shane (1994) have reported that classical IBDV strains vary in virulence, consisting of mild to intermediate strains causing bursal damage and mortality up to 30%. Meanwhile, variant IBDV strains are antigenically distinct that are able to break immunity induced by a conventional classical vaccine (Heine *et al.*, 1991). Chickens infected with typical variant strains usually develop a rapid onset of bursal atrophy without significant inflammation (Rosenberger and Cloud, 1986).

A highly virulent IBDV was first diagnosed in the Netherlands and spread rapidly throughout Europe (Lasher and Shane, 1994). Despite vaccination, highly virulent IBDV caused up to 25% mortality in broilers, 60% in layers and 100% in specific-pathogen-free (SPF) chickens (Van den Berg *et al.*, 1991). Currently, highly virulent strains have been isolated in several

Asian countries such as Japan (Yamaguchi *et al.*, 1997), China (Cao *et al.*, 1998) and Malaysia (Hair-Bejo, 1993). In contrast to the variant strain, these highly virulent strains retain very similar antigenicity to classical strains but vary considerably in virulence. However, recent studies have reported that several highly virulent IBDV strains might be antigenically different due to mutation in the VP2 hypervariable region (Eterradossi *et al.*, 1998; Hoque *et al.*, 2001).

IBDV (species *Infectious bursal disease virus*, genus *Avibirnavirus*, family *Birnaviridae*) has a genome consisting of two segments of double-stranded RNA (dsRNA), A and B (Lasher and Shane, 1994). Segment A is about 3.4 kb and have two open reading frames (ORFs). The long ORF of 3,039 bp encodes a 110K precursor polyprotein (NH_2-VP2-VP4-VP3-COOH) that is processed into mature VP2, VP3 and VP4 (Hudson *et al.*, 1986). Mature VP2 is the major host-protective antigen with epitopes that elicit neutralisation antibodies, while VP3 is a minor host-protective antigen with several group-specific epitopes (Oppling *et al.*, 1991; Yamaguchi *et al.*, 1996). VP4 is a putative protease. The short ORF of 438 bp overlaps with the 5' end of the long ORF and encodes a VP5 of 21K, whose function is not known (Mundt *et al.*, 1995). Segment B is about 2.8 kbp encodes VP1 of 90K, a putative RNA-dependent RNA polymerase (Azad *et al.*, 1985).

A number of field and laboratory strains of IBDV have been characterised molecularly. Two of the most widely used methods are

reverse-transcriptase / polymerase chain reaction–restriction fragment length polymorphism (RT-PCR-RFLP) and sequence analysis. The common area of investigation is the hypervariable region of VP2 gene that codes for the immunodominant viral epitopes (Becht *et al.*, 1988; Fahey *et al.*, 1989). Although RT-PCR-RFLP analysis revealed the existence of genetic variation among most of the IBDV isolate, they are not correlated with subtypes (Maizan *et al.*, 1997). Viruses within the same subtype sometimes have different RFLP and conversely, viruses within different subtypes had identical RFLP (Ismail *et al.*, 1988). A study by Jackwood and Sommer (1998) also found that their RT/PCR-RFLP assay did not provide information on the virulence of the IBDV strains and the results can only be used as predictor of IBDV antigenicity.

A more informative approach for gene characterisation is through sequence analysis. Furthermore, vast information can be obtained by direct analysing the sequence with different bioinformatic tools such as the restriction enzyme cutting sites, mutation sites, evolution and relationship of different strains through phylogenetic analysis, prediction of protein structure that are related to the functions and other special features of the protein. Several studies have shown that the nucleotide and amino acid sequence changes in IBDV were correlated with antigenicity (Heine *et al.*, 1991; Jackwood and Jackwood, 1994; Vakharia *et al.*, 1994) and pathogenicity (Lin *et al.*, 1993; Yamaguchi *et al.*, 1996). The VP2 gene, in particular the hypervariable region was chosen for sequence analysis in many reported

IBDV strains (Brown *et al.*, 1994, Lin *et al.*, 1993; Dormitorio *et al.*, 1997). However, determination of the sequence of whole segment A which consists of VP2, VP3, VP4 and VP5 provides more complete information in the attempt to detect unique amino acid specific to isolate and strain as well as to identify determinants responsible for virulence enhancement (Brown, *et al.*, 1996; Yamaguchi *et al.*, 1997).

Currently, the only way to control the IBDV is by proper sanitation management and vaccination of breeder flocks and their progeny. The most commonly used vaccines are live attenuated and killed vaccines, but they have several disadvantages. One of the major disadvantage is that the intermediate or hot live vaccine used for control of field highly virulent IBDV can induce immunosuppression and not 100% protective. Furthermore, despite recent advances in vaccination programmes, outbreaks of IBDV still occur worldwide. In Malaysia, several outbreaks had occurred between 1991 and 1997. It cannot be denied that local poultry farms depend totally on imported biologics such as vaccines, diagnostic kits, feed supplements and many others. These approaches may prove inadequate for the rapidly developing poultry industry in Malaysia to achieve long-term stability and self-sustainability. Thus, development of new vaccines derived from local IBDV strain is an important area of IBDV research in Malaysia. Application of recombinant DNA technology has made possible the identification of VP2 gene of IBDV which is crucial for inducing protective immune response (Fahey *et al.*, 1991). Taking advantage of this fact, the production of vaccines

from infectious agent which would not replicate as the pathogenic agent but able to induce immunity without causing any risk of increased or altered pathogenicity upon exposure to infection may bring about more effective vaccine.

IBD has been an acute important disease of poultry farms in Malaysia since 1990's. In this study, an attempt was made to develop a recombinant vaccine using our local isolate in the hope that it could cater for a more effective improvement of viral disease control cause by the outbreak of local strains. The first step of the study is to characterise a local strain that can be used to develop the vaccine molecularly which is still lacking currently. In addition, knowledge on the features of the VP2 protein structure would be an advantage since the VP2 gene was used to construct the recombinant vaccine.

Therefore, the objectives of this project can be summarised as below:

1) to study the genetic basis and structure of VP2 gene through sequencing and bioinformative tools;
2) to determine the relationship of UPM97/61 with other strains through phylogenetic analysis of VP2, VP3, VP4 and VP5 gene;
3) to produce the VP2 recombinant protein through expression of the gene in *E. coli* cells; and
4) to investigate the efficacy of the VP2 expressed protein against highly virulent strains of IBDV.

CHAPTER II

LITERATURE REVIEW

Infectious Bursa Disease Virus and The Disease

Taxonomic Classification

Infectious bursal disease virus (IBDV) is a double-stranded RNA virus infecting vertebrates, that is classified under the family Birnaviridae. Since it infects only birds, it is designated as a genus of avibirnavirus (Dobos *et al.*, 1979). The taxonomic classification of IBDV is summarised in Table 2.1.

Table 2.1: Taxonomic classification of IBDV

Family	:	Birnaviridae
Genus	:	*Avibirnavirus*
Species	:	Infectious bursal disease virus
Strains	:	Classical (eg., STC; D00499)
		Variant (eg., variant E; A
		Highly virulent (eg., UPM97/61; AF247006)

Virus Strains

Two serotypes of IBDV, serotype 1 and serotype 2 have been recognized. Serotype 1 is pathogenic to chicken whereas serotype 2 which was isolated from turkey (Mcferran, *et al.*, 1980; Jackwood *et al.*, 1982; Cummings *et al.*, 1986) is non-pathogenic for both turkeys and chickens

(Ismail *et al.*, 1987; Lasher and Shane, 1994). Based on the antigenic variation and virulence, serotype 1 strains can be divided into classical strains, antigenic variant strains and very virulent (vv) or highly virulent (hv) strains.

The classical IBDV strain was recognised in 1972. These strains varied in virulence, consisting of mild to intermediate strains, causing bursal damage and mortality up to 30% (Saif *et al.*, 1987; Lukert and Saif, 1997).

The antigenic variant strains were isolated in the Delmarva region of United States (Rosenberger and Cloud, 1986) in the mid 1980`s. These virus strains are responsible for vaccination failures and can cause immunosuppression in flock vaccinated with classical serotype 1 vaccines (Snyder *et al.*, 1988; Bayyari *et al.*, 1996). Subsequent studies indicated that the variant strains were both antigenically and pathotypically distinct from the original classical strains (Becht *et al.*, 1988; Jackwood and Saif; 1987; Rosales *et al.*, 1989; Ture *et al.*, 1993). These variants have now been shown to occur in many countries around the world (Cao *et al.*, 1998).

The highly pathogenic strain of IBDV was isolated from several farms in Europe in the late 1980's including the Netherlands in 1986 (Box, 1989), Belgium in 1987 (van den Berg *et al.*, 1991) and Great Britain in 1988 (Chettle et al., 1989). This strain was called "very virulent" (vv) or "highly virulent" (hv) as it caused high flock mortalities up to 60% for layer, 25% for